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13. ABSTRACT *(Maximum 200 Words)*

Cancer is essentially a disease of inappropriate proliferation due to a loss of normal cell cycle controls. The scope of this work was to study how cell cycle regulation by G1-phase Cyclin dependent kinases (Cdks) relates to breast cancer causation. The purpose of this proposal was to use yeast as an experimental system to examine the role of G1-Cdks in promoting cell cycle progression. The three major findings of this proposal are detailed below. First, we have shown that G1 cyclins are constitutively unstable and rate limiting for cell cycle progression [1]. Second, the only essential function of G1 cyclins is to phosphorylate the B-type Cdk inhibitor, Sic1, and target it for degradation [2]. Third, Sic1 is one of few known in vivo substrates of yeast G1-Cdks [3]. Sic1 mutants lacking several phosphorylation sites are stabilized and completely block cell cycle progression [1,3]. Thus, the amount and phosphorylation state of Sic1 appears to determine the timing and size of cell division [3]. By studying the mechanism of cell cycle control, we can begin to understand how cell cycle defects leads to abnormal proliferation, and how by preventing inappropriate proliferation, we may be able to reduce the incidence of breast cancers.

14. SUBJECT TERMS
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Introduction

In the three years that this proposal was funded, more than half a million women developed breast cancer and nearly one quarter of these women died from breast cancer [4]. In the simplest sense, cancer is a major killer because cells divide when they should not. There is a very strong link between cell cycle regulation and breast cancer. Overexpression of G1-phase cyclins promotes inappropriate cell division and appears to have a causative role in cancer causation [5-9]. Understanding how cells coordinate cell growth with cell division is fundamental to elucidating the mechanism of cancer causation. The majority of cells in the human body are capable of dividing, but remain quiescent in the absence of mitogenic stimuli. Cancer cells are not bound by this limitation and proliferate in the absence of mitogens by overexpressing the oncogenes that mitogenic stimuli up-regulate (e.g. G1-phase cyclins: D-type cyclins in humans and Clns in yeast). However, while overexpression of G1-phase cyclins commits cells to divide, it does not change the growth rate of cells. Continued cell division in the absence of increased cell growth is disastrous. Thus, cancer cells must also increase their rate of cell growth (e.g., the rate of protein synthesis). We use the yeast *S. cerevisiae* to study the relationship between cell division controls and cancer. Remarkably, the basic cell cycle machinery is so conserved between yeast and humans that human cyclin D1 functions seamlessly in yeast [10-12]. This conservation of function and the genetic tractability of yeast champion it as an excellent model system to study cell cycle regulation and its relation to human carcinogenesis.

Body

Technical Objectives:

1. *Characterization of Sic1 turnover.*
    a. *Examine Sic1 abundance and half-life as a function of cell cycle position.*
    b. *Examine the half-life of Sic1 as a function of the presence of Cln.*
    c. *Mutate the potential Cdc28 phosphorylation sites of Sic1 to see if these contribute to turnover.*

2. *Does Sic1 set the Cln threshold?*
    a. *Measure Sic1 levels at different growth rates and correlate to Cln levels.*
    b. *Titrate Sic1 against Clns. Is more cln need for Start if more Sic1 is present?*

3. *Find mutants which uncouple budding from Start*

Specific Aim #1

The goal for the first specific aim was to show that the abundance of Sic1 varies with cell cycle position and that phosphorylation by Cln-Cdc28 kinase complexes regulates Sic1 stability. We have demonstrated that Sic1 levels are highest in unbudded G1 cells. As cells begin to traverse Start, a slower migrating form of Sic1 becomes apparent, and then Sic1 levels rapidly diminish [3]. Experiments suggest that the slower migrating form of Sic1 is due to phosphorylation presumably by Cln-Cdc28 kinases [2]. However, recent reports indicate that Pho85 kinase complexes can phosphorylate Sic1 [13].

Sic1 abundance has also been measured as a function of Cln abundance. Using both asynchronous cultures and synchronized cultures, we have been able to demonstrate an inverse relationship between Cln abundance and Sic1 abundance [3]. When Cln levels are low, Sic1 levels are high and vice versa (see also aim #2). This strongly suggests that Cln levels regulate Sic1 levels. Sic1 is an excellent in vitro substrate for Cln-Cdc28 complexes possessing as many as 14 different phosporylation sites [3]. Substitution of alanine for serine or threonine in two or three of these sites greatly stabilizes Sic1 [3, 14], and overexpression of these stabilized forms of Sic1 results in a G1-like

cell cycle arrest [3, 14]. This is in agreement with our previous observation that the essential function of Cln-Cdc28 kinases is to phosphorylate Sic1 and target it for degradation. Others and we have established that the essential role of Cln-Cdc28 kinase complexes is to phosphorylate and target Sic1 for degradation [2, 14-19]. It is highly likely that the cumulative effect of these phosphorylations is a general decrease in the half-life of Sic1. It is worth noting that while all Cln-Cdc28 complexes appear to be able to phosphorylate Sic1 in vitro, cells possessing only the Cln3-Cdc28 kinase complex (e.g. *cln1 cln2 clb5 clb6*) mutants appear to be unable to direct the degradation of Sic1 [3]. This suggests that Sic1 phosphorylations may be qualitatively different.

Specific Aim #2

Both human and yeast cells require high protein synthesis rates and a minimum cell size requirement for cell division. Our research has demonstrated that these two requirements are intricately related [3]. We have shown that bulk protein synthesis rates are directly proportional to cell size, and that large cells support higher proliferation rates than small cells [3]. Further, our research has shown that G1-phase cyclins (Clns) are unstable rate limiting activators of cell division [1]. Like cyclin D1 in humans, yeast G1-phase cyclins (Clns) are exquisitely sensitive to protein synthesis rates and act as a metric of both cell size and cell growth rate. In humans G1-phase Cdks (e.g., cyclin D1 Cdk complexes) promote cell division by phosphorylating and inactivating the RB tumor suppressor gene [20-22]. In yeast, G1-phase Cdks phosphorylate and inactivate Sic1 to promote cell division [2]. Our research has shown that cells lacking Sic1, like cells lacking RB, no longer require G1-phase Cdks for cell division [2]. Sic1 acts analogously to pRb. Finally, we have shown that like pRb, the phosphorylation state of Sic1 correlates well with the proliferative capacity of cells. Hyperphosphorylated Sicr1 is rapidly degraded, while hypo-phosphorylated Sic1 blocks cell division [3].

The goal for this specific aim was to determine whether Sic1 levels vary with the growth rate of the culture and determine whether Sic1 sets the Cln threshold for Start. We have shown that cells possessing an extra copy of Sic1 appear to delay initiation of S-phase and may require more Cln to commit to cell cycle progression than control cells [3]. Thus, it appears that underphosphorylated Sic1 sets the Cln threshold for Start. This places Sic1 at the heart of commitment to cell cycle progression and more firmly establishes its role as acting analogously to the RB1 gene in higher eukaryotes.

Specific Aim #3

We have shown that Sic1 couples S-phase to Start [2]. In fact, Sic1 appears to intrinsically regulate all aspects of Start [3]. In wild type cells, Cln-Cdc28 initiates S-phase by phosphorylating Sic1 and targeting it for degradation [2,19]. Initiation of the budding process, which is analogous to determining the plan of division, occurs at the same time, but a yet unknown mechanism. Maybe Cln-Cdc28 also inhibits an inhibitor of budding. The goal of this specific aim was to identify yeast mutants that are able to bud in the absence or near absence of Cln. In the past year, we have cloned all the required constructs necessary for this objective and have begun to screen for the desired mutants. Briefly, to attempt to identify and clone such an inhibitor, we have mutagenized a conditional Cln strain and looked for cells that are able to bud in the absence or near absence of Cln. We have identified two classes of mutants, one class of mutants that appear to bud in the complete absence of Cln activity and one class that requires less Cln to bud than wild type cells. We are currently investigating these mutants and attempting to clone members of each type of mutant. This screen is far from saturated, and all indications are that this ongoing genetic screen will yield a wealth of valuable and interesting mutants.

Future Plans:

The abundance of Sic1 has been measured with respect to cell cycle position, growth rate, and Cln levels, but there is still a need to address the issue of the half-life of Sic1. It is not known in detail how the presence or absence of Cln-Cdc28 kinase activity affects the half-life of Sic1. While we have

indications that lower growth rates increase the half-life of Sic1, this needs to be measured precisely. While the kinases responsible for phosphorylating Sic1 and pRb are known, the missing pieces in the cell cycle puzzle are the protein phosphatases that dephosphorylate RB or Sic1

Recently, Sic1 has been shown to be a substrate for the Cdc14 protein phosphatase [23]. Further, it has been shown that the activity of the PTEN tumor suppressor gene, itself a protein phosphatase, correlates with the phosphorylation state of the RB protein. Exciting recent research has pushed two protein phosphatases to the forefront: PTEN and Cdc14. Inactivation of the PTEN tumor suppressor gene in *Drosophila* stimulates protein synthesis and dramatically increases cell growth and the proliferative capacity of cells [24-26]. Moreover, overexpression of PTEN in PTEN-/- cancer cell lines greatly reduces pRb phosphorylation and induces a rapid G1 arrest [24-26]. The Cdc14 protein phosphatase is intricately involved in cell cycle progression in yeast [23]. Because mutations of PTEN and pRb tumor suppressor genes have been found in a large fraction of breast cancers, there is a clear need to further elucidate the role of PTEN/Tep1 and Cdc14 in cell cycle progression and to determine whether these phosphatases modulate the amount of G1-phase Cdk activity required for cell division. Because the PTEN and RB genes are clearly two of the most significant breast cancer related genes known today, we have recently submitted a grant to the Breast Cancer Research Program at the Department of Defense for the fiscal year 2001 to investigate the role of these genes in cell cycle progression in yeast. If this grant is funded we will pursue the following questions: What is the phosphatase that dephosphorylates Sic1? Do the levels of this phosphatase determine the levels of Cln required for cell cycle progression? How stable is Sic1 in the complete absence of Cln activity? Do phosphorylation mutants that stabilize Sic1 raise the Cln threshold for Start? Do acidic substitutions destabilize Sic1 and lower the Cln threshold for Start? We have preliminary evidence that the Sic1 protein has a function independent of its kinase inhibitory function, and we attempting to separate these functions to assess the importance of each in cell cycle regulation. Experiments are underway that address all of these questions and we expect the answers to be both very interesting and very elucidating in the molecular mechanism of cell cycle progression.

Conclusions:

The regulation of cell cycle progression is a basic biological problem. However, very little is known about the targets of Cdk phosphorylations, and how commitment to cell division is controlled. We have identified Sic1, a potential analog to the RB tumor suppressor gene, as a key Cdk substrate whose phosphorylation has a central role in regulating proliferation [manuscript enclosed]. Cln proteins like cyclin D/E in mammalian cells are highly unstable rate limiting activators of cell cycle progression [manuscript enclosed]. Cln and Sic1 levels are intricately related, and it is likely that Sic1 acts to restrain cell cycle. The relationship between cell size, cell growth rate, and Cln activity is an ongoing puzzle, and we will continue to investigate these inter-relationships. A better working model of how Sic1 controls cell cycle progression may go a long way towards understanding how cell cycle control effects the onset of cancer

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Key research accomplishments:

=

- *The only essential function of Clns is to phosphorylate and target Sic1 for degradation.*

- *Sic1 abundance is dependent upon cell cycle position, growth rate, and Cln levels.*

•*Sic1 is phosphorylated qualitatively differently by different Cln-Cdk complexes.*

•*Clns are constitutively unstable.*

•*Sic1 abundance and phosphorylation state may determine the timing of Start*

Reportable Outcomes:

•*The work presented here has generated two first author manuscripts.*

Schneider B.L., Patton, E.E., Lanker, S. Mendenhall, M.D., Wittenberg, C., Futcher, B. and Tyers, M. (1998) Yeast G1 cyclins are unstable in G1 phase. *Nature* 395 86-89.

Schneider, B.L., Yang, Q.-H., and Futcher B. (1996) Linkage of replication to Start by the Cdk inhibitor Sic1. *Science* 272, 560-562.

Schneider, B.L. G1 cyclin thresholds link cell division to growth rate. 2000 DOD Era of Hope meeting. Atlanta, Georgia

Research from this award has generated two additional manuscripts which are in preparation and will be submitted by the end of this year.

Grants Funded based on preliminary work supported by this grant

American Heart Association (Schneider) 7/1/2000-6/30/02 $120,000
The role of the cyclin dependent kinase inhibitor Sic1 in transcriptional repression and cell cycle inhibition.
The major goals of this project are to identify genes and promoter elements responsible for transcriptional repression of G1-phase genes, and to examine the role of Sic1 in this mechanism.

Wendy Will Case (Schneider) 7/1/00-6/30/01 $25,000
G1 Cyclins and Transcription: The Link Between Sic1, Transcriptional Repression and Cancer
The major goals of this project are to identify the mechanism whereby G1-Cdks in yeast repress the transcription of genes required for cell division, and to assess the role of the Cdki, Sic1, in this mechanism.

South Plains Foundation 9/1/00-8/31/01 $10,000
The Role of cyclin-Cdk complexes in promoting Meiosis
The major goals of this project are to identify the roles of Cdks in meiosis.

Southwest Cancer Center Seed Grant 9/1/00-8/31/01 $7,000
The role of Sic1 in commitment to cell division.
The major goals of this proposal are to determine how the abundance, the activity, and the half-life of Sic1 are regulated by its phosphorylation state, by growth rate, and by cell cycle position.

CH Foundation 12/1/00-11/30/01 $20,000
Novel mechanisms for preventing inappropriate proliferation.
The major goals of this project are to identify genes that inhibit cell division.

Previously funded grants

TTUHSC (Schneider) 9/1/1999 – 8/31/2000 $20,000
Divide or Bust: Genes That Regulate Cell Division in Yeast

South Plains Foundation (Schneider) 9/1/1999 - 08/31/2000 $10,000
Cancer and Cell Cycle Progression: A Link to Gene Expression

•*In the course of this award, I have been hired as an assistant professor in the department of Cell Biology and Biochemistry at Texas Tech Health Sciences Center*

Yeast G1 cyclins are unstable in G1 phase

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* *These authors contributed equally to this work.*

In most eukaryotes, commitment to cell division occurs in late G1 phase at an event called Start in the yeast *Saccharomyces cerevisiae*[1], and called the restriction point in mammalian cells[2]. Start is triggered by the cyclin-dependent kinase Cdc28 and three rate-limiting activators, the G1 cyclins Cln1, Cln2 and Cln3 (ref. 3). Cyclin accumulation in G1 is driven in part by the cell-cycle-regulated transcription of *CLN1* and *CLN2*, which peaks at Start[3]. *CLN* transcription is modulated by physiological signals that regulate G1 progression[4,5], but it is unclear whether Cln protein stability is cell-cycle-regulated. It has been suggested that once cells pass Start, Cln proteolysis is triggered by the mitotic cyclins Clb1, 2, 3 and 4 (ref. 6). But here we show that G1 cyclins are unstable in G1 phase, and that Clb–Cdc28 activity is not needed for G1 cyclin turnover. Cln instability thus provides a means to couple Cln–Cdc28 activity to transcriptional regulation and protein synthetic rate in pre-Start G1 cells.

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Cln1, Cln2 and Cln3 are non-abundant proteins that efficiently promote the G1/S transition. Direct measurement of Cln half-lives in G1 phase has been difficult. The Clns are very unstable proteins with half-lives of less than 10 min in asynchronous populations[7–14], consistent with the idea that Cln instability is constitutive. Indeed, Clns are unstable in G1 cells arrested with mating pheromone[9,15,16], but this could be a special effect of pheromone rather than being representative of G1 cells in logarithmic growth. However, it is possible that Cln1 and Cln2 are stable in late G1 and are degraded shortly after cells pass through Start.

To examine the G1-phase stability of Cln2 directly, we measured the half-life of Cln2 in small G1-phase cells obtained by elutriation. We used strains that contained either $CLN2^{HA}$ (which is phenotypically indistinguishable from untagged *CLN2* (see Fig. 1b)) or $CLN2^{4T3S\text{-}HA}$, both expressed from the *GAL1* promoter. $Cln2^{4T3S}$ is a multiple point mutant that lacks seven consensus Cdc28 phosphorylation sites and is sevenfold more stable than wild-type Cln2 in asynchronous cultures[12]. The half-life of the mutant and wild-type proteins were measured in G1 cells by decay of the Cln2 signal after repression of the *GAL1* promoter by glucose. Wild-type Cln2 had a half-life of 5–10 min in the G1 cells (Fig. 1a), which remained in G1 phase throughout the experiment. In contrast, the stabilized mutant $Cln2^{4T3S}$ had a longer half-life, and a fraction of the cells budded and began DNA synthesis (Fig. 1a). This experiment shows directly that Cln2 is unstable in G1 phase and suggests that Cln2 instability is required for proper regulation of Start.

One caveat to this experiment is that the instability of Cln2 might have been due partly to the HA epitope tag, which was required to detect the low levels of wild-type Cln2 in small G1 cells. However, we compared the stability of HA-tagged versus untagged Cln2 and found that the half-lives of these two forms of Cln2 were indistinguishable in asynchronous cultures (Fig. 1b). We also determined that neither tag position nor the epitope sequence altered Cln2 stability (not shown). Furthermore, we compared the ability of untagged *GAL1–CLN2* and untagged $GAL1\text{–}CLN2^{4T3S}$ to promote Start. Small G1 cells were obtained by elutriation, and then the *GAL1* promoter was turned on slightly with a low concentration of galactose in a semi-repressing medium. G1 expression of $Cln2^{4T3S}$ accelerated Start and reduced critical cell size compared to the expression of wild-type Cln2 (Fig. 1c). The differential ability of stabilized Cln2 to promote Start again suggests that wild-type Cln2 is unstable in G1 cells.

It has recently been suggested that the Clb mitotic cyclins are necessary for Cln degradation[6], a model that predicts that G1 cyclins should be stable in pre-Start G1 cells because such cells lack Clb protein entirely[17]. Because this prediction is inconsistent with our finding that Cln2 is unstable in G1 cells, we measured Cln2 stability under three different conditions that severely reduce or eliminate Clb–Cdc28 activity. Cells lacking Clb1 to Clb4 (strains MT383 and K3390, which we call '$clb1\text{–}4^{ts}$')[18] arrest in G2 phase, whereas cells lacking Clb1 to Clb6 (strain K4057, which we call '$clb1\text{–}6^{ts}$')[19] arrest in G1 phase. Cells that overexpress the Clb–Cdc28 inhibitor Sic1 also accumulate in G1 phase[20,21]. When Cln stability was measured in a $clb1\text{–}4^{ts}$ strain by repression of a $GAL1\text{–}CLN2^{HA}$ construct (Fig. 2a), or a $GAL1\text{–}CLN1^{HA}$ construct (not shown), Cln was no

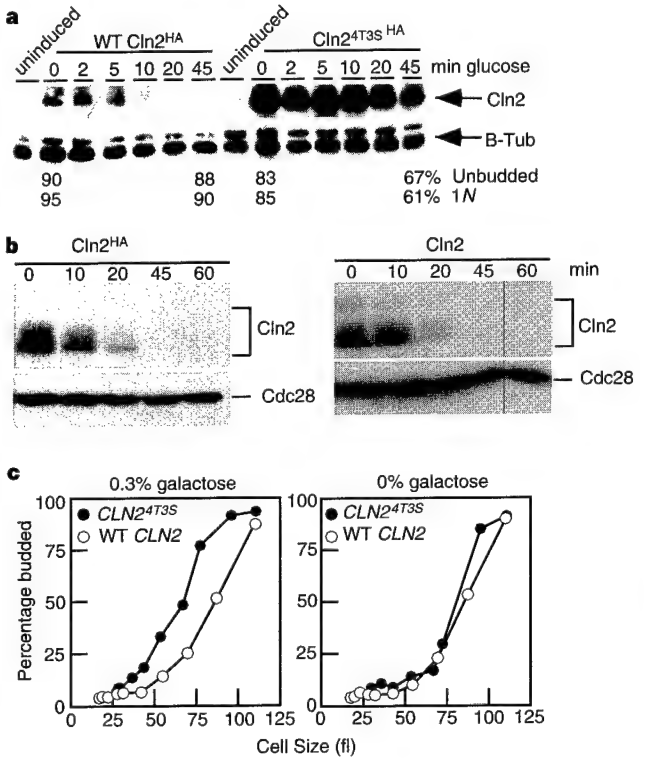

Figure 1 Cln2 is unstable in G1 phase. **a**, Direct measurement of Cln2 stability in G1 cells. The decay of wild-type (WT) $Cln2^{HA}$ or the stable mutant $Cln2^{4T3S}$ in synchronized G1-phase cells after repression of weakly induced *GAL1–CLN2* by glucose was measured by immunoblotting against the HA epitope. Cells in G1 phase were assayed as the percentage of unbudded cells, and as the percentage of cells with 1*N* DNA content. B-tubulin immunoreactivity was used as a loading control. **b**, The HA epitope tag does not affect the stability of Cln2. Tagged Cln2 and untagged Cln2 were expressed from the *GAL1* promoter in asynchronous cultures. Cln2 decay after repression of the *GAL1* constructs by 2% glucose was assessed by immunoblotting with an anti-Cln2 rabbit polyclonal antibody. **c**, $CLN2^{4T3S}$ (untagged) is a better inducer of Start than wild-type *CLN2* (untagged). $GAL1\text{-}CLN2^{4T3S}$ or *GAL1–CLN2* was weakly induced in small G1 cells and the rate of passage through Start was assessed by bud formation as a function of cell size.

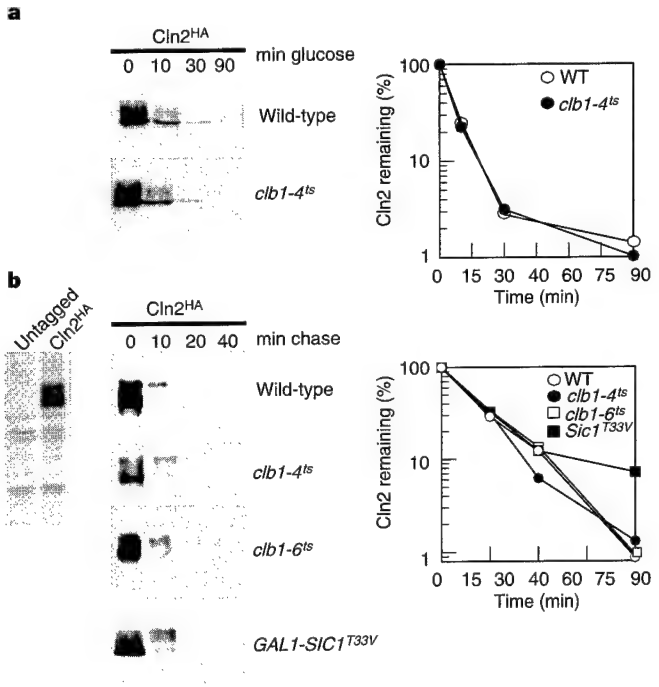

Figure 2 Clb activity is not required for Cln2 degradation. **a**, Cln2 is unstable in a $clb1\text{–}4^{ts}$ strain. $GAL1\text{-}CLN2^{HA}$ was expressed in a wild-type (WT) or $clb1\text{–}4^{ts}$ strain at a non-permissive temperature, and repressed by glucose for the indicated times. $CLN2^{HA}$ immunoreactivity was quantified by densitometry and normalized to Cdc28 immunoreactivity on the same blot. **b**, Cln2 is unstable under three different conditions in which Clb activity is severely compromised. $CLN2^{HA}$ was expressed at wild-type levels from the *CLN2* promoter in $clb1\text{–}4^{ts}$, $clb1\text{–}6^{ts}$ and $GAL1\text{-}SIC1^{T33V}$ strains under non-permissive conditions. Stability of $Cln2^{HA}$ was determined by [^{35}S]methionine/cysteine pulse-chase analysis.

more stable than in wild-type cells. To rule out possible effects of *CLN* overexpression and carbon source shifts on Cln stability, we performed pulse–chase analysis of ^{35}S-labelled Cln2 expressed at wild-type levels from its own promoter. Cln2 was as unstable in the *clb1–4^{ts}* and *clb1–6^{ts}* arrested cells as in the wild-type strain (Fig. 2b). Cln2 was also unstable in cells overexpressing *SIC1^{T33V}*, which encodes a partly stabilized version of Sic1 (Fig. 2b). Furthermore, we failed to detect any alteration in Cln1 half-life in cells arrested by overexpression of *SIC1^{T45A}* (not shown), which encodes a highly stable version of Sic1. Therefore, the mitotic cyclins cannot be essential for G1 cyclin degradation.

Finally, we re-examined the role of the ubiquitin-conjugating enzyme Cdc34 in the turnover of Cln2. There is persuasive evidence that Cdc34 has a direct role in conjugating ubiquitin to Cln2 (refs. 11, 14) and to Sic1 (refs 20, 22, 23), leading to degradation. For ubiquitination of Sic1, Cdc34 acts in concert with an E3 ubiquitin protein ligase complex composed of Skp1, Cdc53 and the F-box protein Cdc4 (the SCF^{Cdc4} complex)[22,23]. Cln ubiquitination proceeds via a similar pathway, but depends on a different F-box protein, Grr1, which forms the analogous SCF^{Grr1} complex[13,22,24]. However, it has been argued that Cdc34 promotes Cln degradation indirectly by promoting the degradation of Sic1, thereby activating Clb kinases[6]. If this were true, then *cdc4* mutants should also be defective in Cln2 turnover, because Sic1 accumulates in *cdc4* mutants just as it does in *cdc34* mutants[19]. However, Cln2 degradation was defective only in the *cdc34* mutant (Fig. 3a), despite the fact that Sic1 accumulated in both the *cdc34* mutant and the *cdc4* mutant (Fig. 3b). The fact that the *cdc34* arrest phenotype is indistinguishable from *cdc4* and *clb1–6^{ts}* arrests, yet Cln2 is stabilized only in the *cdc34* arrest, indicates that Cln stabilization in *cdc34* mutants is not an indirect effect of Sic1 accumulation and loss of Clb activity.

A model in which G1 cyclins are stable in G1 phase and then catastrophically destroyed as cells pass Start, is attractive because it is analogous to the cell-cycle-regulated destruction of mitotic cyclins in anaphase[17]. However, our data rule out this model and support a model in which Cln proteins are constitutively unstable. On the basis of our data we suggest that the rise in Cln abundance is due to a rise in the rate of Cln synthesis, which depends both on a rise in *CLN* transcription and on increases in overall protein synthesis as cells grow. After Start, the onset of mitotic cyclin activity represses Cln synthesis, but only at the transcriptional level[18]. The instability of the Cln proteins probably derives from Cdc28-dependent autophosphorylation of the Cln subunit, which targets the Cln for degradation[10–12,14]. The intrinsic instability of the Cln proteins is advantageous for the cell because it allows almost instantaneous responses to changes in the rate of protein synthesis[25] and *CLN* transcription[4,5], both of which are key determinants of whether or not cell division is appropriate. The continuous requirements for growth factors and protein synthesis in G1-phase progression of mammalian cells[26], and the phosphorylation-dependent instability of cyclin D and cyclin E (refs 27–29), suggest that a similar control of G1 cyclin activity operates in mammalian cells. □

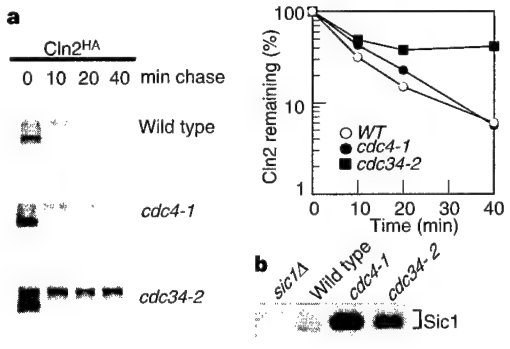

Figure 3 Sic1 accumulation does not affect Cln2 stability. **a**, Cdc34, but not Cdc4, function is required for Cln2 degradation. *CLN2^{HA}* was expressed at wild-type levels from the *CLN2* promoter in wild-type, *cdc4-1* and *cdc34-2* strains at the non-permissive temperature. Stability of Cln2^{HA} was determined by [^{35}S]methionine/cysteine pulse-chase analysis. **b**, Abundance of Sic1 was determined in lysates from each strain in **a** by immunoblotting with anti-Sic1 antibody.

Methods

Cell synchronization. All strains were isogenic with W303 (*adc2 his3 leu2 trp1 URA3*). To determine the half-life of Cln2 in G1 cells, strain BS328 (*cln3*) containing either plasmid pSL46 (ref. 12) (*GAL1–CLN2^{HA} URA3 CEN*, strain BS333) or plasmid pSL122 (ref. 12) (*GAL1–CLN2$^{4T3S\text{-}HA}$ URA3 CEN*, strain BS322) was grown to early log phase in yeast extract peptone medium plus 2% sucrose. The use of a *cln3* deletion strain, which has a prolonged G1 phase[15], was required to allow sufficient time for half-life time courses to be completed before cells passed Start. Small G1 cells were obtained by elutriation at room temperature in clarified medium. The *GAL1* promoter was weakly induced for 1 h by the addition of galactose in the presence of 2% sucrose (a semi-repressing condition) and then repressed by the addition of 2% glucose. The decay of Cln2^{HA} or Cln2$^{4T3S\text{-}HA}$ was assayed by immunoblotting as described[15].

To assess the ability of Cln2 to activate Start, strain BS328 containing plasmid pB75 (*GAL1–CLN2 URA3 CEN*, strain BS412) or plasmid pB101 (*GAL1–CLN2^{4T3S} URA3 CEN*, strain BS410) were grown and elutriated as above. After G1 cells were obtained, galactose (0.3%) was either added or not added in the presence of 2% sucrose. Passage through Start was assessed by monitoring bud formation by microscopy, and modal cell size with a Coulter Channelyzer[15].

Protein analysis. For determination of Cln2 half-life by promoter shut-off, strain MT386 (*cln2::GAL1–CLN2^{HA}–LEU2*) and an isogenic *clb1-4^{ts}* strain MT383 (*clb1 clb2-VI clb3::TRP1 clb4::HIS3 cln2::GAL1–CLN2^{HA}–LEU2*)[18] were grown in 2% raffinose, shifted to 37 °C for 2 h, induced with 2% galactose for 1.5 h at 37 °C, then repressed with 2% glucose at 37 °C for the indicated times. Similar results were also obtained at a lower non-permissive temperature of 34 °C. Cln2 decay was assayed by immunoblotting with 12CA5 antibody using detection by enhanced chemoluminescence as described[15]. Polyclonal antibodies directed against Sic1, Cln2, Cdc28 and B-tubulin were used as described[15,22,30].

For determination of Cln2 half-life by ^{35}S pulse–chase analysis, all strains contained a single copy of *CLN2^{HA}* expressed from the *CLN2* promoter at wild-type levels. Strains used were: K3389 (*CLN2^{HA}*)[18], K3390 (*clb1 clb2-VI clb3 clb4 CLN2^{HA}*)[18], K4057 (*clb1 clb2-VI clb3::TRP1 clb4::HIS3 clb5::GAL–CLB5–URA3 clb6::LEU2*)[19], MT668 (*cdc4-1*) and MT670 (*cdc34-2*). K4057 was transformed with plasmid pMT1634 (*CLN2^{HA} ADE2 CEN*); K3389 was transformed with plasmid pMDM202 (*GAL1–SIC1^{T33V} LEU2 CEN*); W303, MT668 and MT670 were transformed with plasmid pMT291 (*CLN2^{HA} LEU2 CEN*). For experiments with temperature-sensitive strains, cultures were shifted to 37 °C for 2 h before the pulse–chase regimen. Strain K4057, which requires induction of *GAL1–CLB5* with 0.3% galactose for viability[19], was simultaneously shifted to glucose medium at the time of the shift to 37 °C. *CLN2^{HA}* stability in strain K3389 was measured at 30 °C after induction of *GAL1–SIC1^{T33V}* with 2% galactose for 2 h.

The pulse–chase regimen, protein extraction and immunoprecipitation were based on ref. 17. In brief, 15 ml of culture at 0.5×10^7 cells ml^{-1} in rich medium supplemented with 0.1 mM methionine was filtered and resuspended in 1 ml of –Met synthetic medium. After 5 min, cultures were pulsed with 500 μCi of [^{35}S]Met/Cys (ICN, Tran ^{35}S-label) and after an additional 5 min cultures were chased with 2 mM unlabelled Met, 2 mM unlabelled Cys, and 1 mg ml^{-1} cycloheximide. Cells were pelleted, resuspended in 5% cold trichloroacetic acid (TCA), washed once with cold acetone and stored at –80 °C. Frozen pellets were resuspended in 75 μl of cold breaking buffer (50 mM Tris-HCl, pH 7.5, 1 mM EDTA, and protease inhibitors: 1 mM PMSF, 0.6 mM dimethylaminopurine, 1 μg ml^{-1} leupeptin, 1 μg ml^{-1} pepstatin, 10 μg ml^{-1} TPCK, 10 μg ml^{-1} soybean trypsin inhibitor) with glass beads and broken by vortex mixing, after which SDS was added to 1% and the lysates boiled for 2 min. Lysates were diluted to 1 ml with 900 μl RIPA buffer (50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 150 mM NaCl, 0.5% sodium deoxycholate, 1% NP-40 and protease inhibitors) plus 0.2 mg ml^{-1} BSA and cell debris was pelleted. We determined that the above lysis buffers were critical to maintain the Cln phosphoisoforms when compared with other lysis buffers[6]. Incorporation of

^{35}S was measured by precipitation with TCA, and equal radioactive counts were used for each immunoprecipitation. The supernatant was precleared with 20 μl protein A–Sepharose beads for 20 min, incubated with 1 μg 12CA5 anti-HA antibody for 1 h and immune complexes collected on 10 μl protein A–Sepharose beads for 2 h. Beads were washed twice with 1 ml RIPA buffer, twice with 1 ml RIPA buffer plus 1% β-mercaptoethanol, and twice with 1 ml RIPA buffer plus 1% β-mercaptoethanol plus 2 M urea. Beads were transferred to a fresh tube after every second wash. Beads were aspirated dry, resuspended in 10 μl cold 2× Laemmli sample buffer, boiled for 2 min and proteins were separated on a 10% SDS–PAGE gel. Radioactive species were visualized and quantitated using a PhosphorImager (Molecular Dynamics). Incorporation into Cln2-specific species was normalized to total radioactivity in parallel lanes of total lysate.

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Linkage of Replication to Start by the Cdk Inhibitor Sic1

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In *Saccharomyces cerevisiae*, three G_1 cyclins (Clns) are important for Start, the event committing cells to division. Sic1, an inhibitor of Clb-Cdc28 kinases, became phosphorylated at Start, and this phosphorylation depended on the activity of Clns. Sic1 was subsequently lost, which depended on the activity of Clns and the ubiquitin-conjugating enzyme Cdc34. Inactivation of Sic1 was the only nonredundant essential function of Clns, because a *sic1* deletion rescued the inviability of the *cln1 cln2 cln3* triple mutant. In *sic1* mutants, DNA replication became uncoupled from budding. Thus, Sic1 may be a substrate of Cln-Cdc28 complexes, and phosphorylation and proteolysis of Sic1 may regulate commitment to replication at Start.

Before yeast can replicate DNA, they must pass Start, which requires a cyclin-dependent protein kinase composed of a catalytic subunit (Cdc28) and one of three G_1 cyclins (Cln1, -2, or -3) (*1*). After Start, B-type cyclin-Cdc28 kinases such as Clb5-Cdc28 and Clb6-Cdc28 must be activated to allow replication (*2*). Although Clb5- and Clb6-Cdc28 complexes are present in G_1 phase, they are initially inactive because of inhibition by the Sic1 protein (*2*, *3*). Activation of Clb5- and Clb6-Cdc28 occurs after Sic1 is targeted for proteolysis by the ubiquitin-conjugating enzyme Cdc34 (*2*). Thus, a *cdc34* mutant arrests with a 1N DNA content because it cannot degrade Sic1, but nevertheless buds, and duplicates its spindle pole body.

It is not known how Start triggers Sic1 inactivation or how replication is tied to other Start-dependent events such as budding and duplication of the spindle pole body. Is Start a single event that affects multiple pathways, or is Start a collection of events, one of which regulates Sic1 proteolysis and replication?

We asked whether Cln-Cdc28 complexes phosphorylate Sic1, thereby targeting it for proteolysis. Sic1 coprecipitates with

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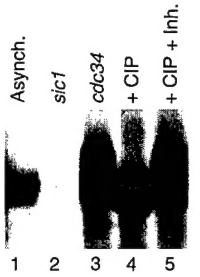

Fig. 1. Sic1 is a phosphoprotein in vivo. Extracts were made as described (*17*), and Sic1 was immunoprecipitated (*14*). The immunoprecipitates were treated or not treated with phosphatase (*18*), resolved by SDS-PAGE (*15*), blotted to nitrocellulose, and Sic1 was detected (*16*). Lane 1, asynchronous cells; lane 2, asynchronous *sic1* cells; lane 3, strain #31 (*19*) arrested at the *cdc34* block at 37°C; lane 4, as in lane 3, but treated with calf intestinal phosphatase (CIP); lane 5, as in lanes 3 and 4, but treated with CIP and the phosphatase inhibitor B-glycerolphosphate (Inh.).

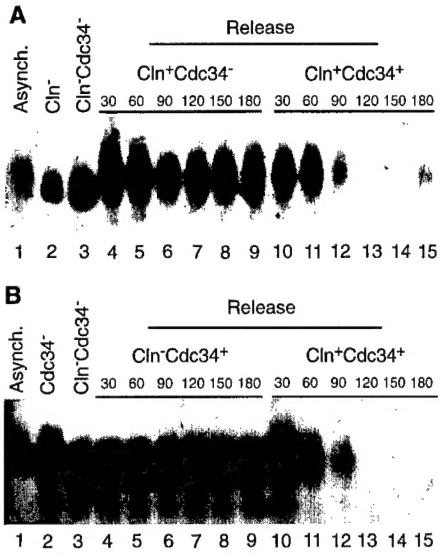

Fig. 2. Loss of Sic1 depends on *CLN*s and on *CDC34*. Abundance and phosphorylation of Sic1 were analyzed in reciprocal shift experiments (*20*). Strain #31 (*cln1 cln2 GAL-CLN3 cdc34*) (*19*) was used. (**A**) Cells were grown in galactose medium at 23°C (lane 1), shifted to glucose at 23°C for 3 hours to synchronize cells at Start (lane 2), then shifted to 37°C for another hour to inactivate Cdc34 (lane 3). Cln expression was then restored by shifting back to galactose medium, but cells were held at 37°C ($Cdc34^-$). Samples were taken every 30 min (lanes 4 to 9). As a control, Cln expression and Cdc34 function were both restored (lanes 10 to 15) to doubly blocked cells. (**B**) Cells were grown in galactose medium at 23°C (lane 1), shifted to 37°C for 3 hours to synchronize cells at the *cdc34* block (lane 2), then shifted to glucose at 37°C for 1 hour to shut off *GAL-CLN3* (lane 3). Cdc34 function was restored by a shift to 23°C, but cells were kept in glucose medium (Cln^-). Samples were taken every 30 min (lanes 4 to 9). As a control, Cdc34 function and Cln expression were both restored (lanes 10 to 15) to doubly blocked cells. FACS analysis showed that the cells in lanes 4 to 9 (A and B) failed to replicate DNA, whereas the cells in lanes 10 to 15 did replicate DNA.

Cdc28 (*4*), has one of the highest densities of potential Cdc28 phosphorylation sites of any known yeast protein (*5*), and can be phosphorylated on many sites by Cdc28 in vitro (*4*, *6*).

Sic1 is a phosphoprotein in vivo. Resolution of Sic1 by SDS–polyacrylamide gel electrophoresis (PAGE) followed by immunoblotting showed a broad, fuzzy band that may contain multiple forms of Sic1. Phosphatase treatment converted this fuzzy band (more phosphorylated form) to a band of greater mobility (less phosphorylated form) (Fig. 1).

To study the relation between the Clns, phosphorylation and proteolysis of Sic1, and DNA synthesis, we constructed a *cln1 cln2 GAL-CLN3 cdc34-2* (temperature-sensitive) strain and did reciprocal shift experiments. As expected, cells shifted from the Cln^-Cdc34^+ state to the Cln^+Cdc34^- state arrested with a $Cdc34^-$ phenotype without dividing. Sic1 accumulated in the less phosphorylated form in Cln^--arrested cells, but was phosphorylated to a greater extent when Cln was restored (*7*) (Fig. 2A, compare lanes 3 and 4). However, in the absence of Cdc34 function (Cln^+Cdc34^-), this highly phosphorylated Sic1 remained undegraded (Fig. 2A, lanes 4 to 9). In control cells arrested in the Cln^-Cdc34^+ state, then released to the Cln^+Cdc34^+ state, Sic1 became more phosphorylated when Cln was restored, and then disappeared, presumably because of proteolysis (*7*) (Fig. 2A, lanes 10 to 15). These cells then reentered a normal cell cycle. Thus, in vivo, the Cln-Cdc28 complexes are needed to generate highly phosphorylated Sic1, which is stable in the absence, but not in the presence, of Cdc34 function.

Cdc34 has been considered to act downstream of Clns and Cdc28. Surprisingly, however, cells shifted from the Cln^+Cdc34^- state to the Cln^-Cdc34^+ state did not enter S phase or divide and in all respects maintained a $Cdc34^-$ phenotype. This result suggests that the Cdc34 function cannot be completed in the absence of Cln-Cdc28 activity. Highly phosphorylated Sic1 accumulated in the Cln^+Cdc34^- cells (*7*) (Fig. 2B, lane 2); Sic1 then became less phosphorylated, but not degraded, after the shift to the Cln^-Cdc34^+ state (*7*) (Fig. 2B, lanes 4 to 9). This result suggests that the $Cdc34^-$ phenotype is maintained in the Cln^-Cdc34^+ cells because the less phosphorylated form of Sic1 cannot be degraded in the absence of Cln activity. When cells were shifted from Cln^+Cdc34^- to Cln^+Cdc34^+, the more phosphorylated form of Sic1 that had accumulated at the *cdc34* block disappeared (Fig. 2B, lanes 10 to 15), and the cells went through S phase and reentered a normal cycle. These experiments show that Sic1 loss requires Cln function as well as Cdc34 function, and that the more phosphorylated form of Sic1 is dependent on Cln activity and correlated with Sic1 loss. Because cells arrest before S phase regardless of the phosphorylation state of Sic1, both forms must inhibit Clb-Cdc28 complexes.

These results are consistent with a model wherein Cln-Cdc28 complexes phosphorylate Sic1, and this phosphorylation targets Sic1 for degradation by the Cdc34 pathway. However, the experiments are correlative, and other mechanisms are also possible. For example, Cln-Cdc28 complexes may serve to activate Cdc34 itself, and the phosphorylation of Sic1 may be a correlated but irrelevant event.

If a major function of Clns is to promote proteolysis of Sic1, then Clns should be less important in a *sic1* mutant. Indeed, a *sic1* mutation suppressed the lethality of a *cln1 cln2 cln3* triple null mutation (Fig. 3B, sectors 1, 3, and 4). Thus, the only nonredundant essential function of the Clns is to inactivate Sic1. The *cln1 cln2 cln3* triple mutation is also suppressed by a mutation

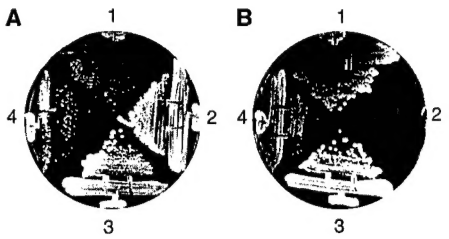

Fig. 3. A *sic1* deletion suppresses lethality of *cln1 cln2 cln3*. (**A**) YEP + 1% raffinose + 1% galactose. (**B**) YEP + 2% glucose. Plates were incubated at 30°C for 3 days. Strains were as follows: 1, BS147 (*pGAL-CLN3 Δclns Δsic1*); 2, BS100 (*GAL-CLN1 Δclns*); 3, BS178 (*GAL-CLN1 Δclns Δsic1*); and 4, BS152 (*Δclns Δsic1*) (*19*).

called *BYC1* (*8*), and it now appears that *BYC1* is allelic to *sic1* (*9*). This suppression by *BYC1* occurs even if *clb2*, *clb5*, or *pcl1* is also deleted (*8*). Clns1, -2, and -3 have other important functions that are compromised in the *cln1 cln2 cln3 sic1* quadruple mutant: Plating efficiency is poor, budding and cell morphology are highly abnormal, and the cells are generally sick. Presumably, budding is now mediated by combinations of other cyclins such as Pcl1, Pcl2, Clb5, and Clb6 (*10*).

If Sic1 is an important and specific inhibitor of replication, then a *sic1* mutation might uncouple DNA replication from other Start events, such as budding. To test this hypothesis, we obtained small unbudded cells from an exponential culture of *sic1* cells and examined the cells for DNA content by fluorescent-activated cell sorting (FACS). At least 20% of the unbudded cells were already 2N, whereas there were essentially no 2N cells in the equivalent fraction from a wild-type culture. After reinoculation into fresh medium, the *sic1* cells

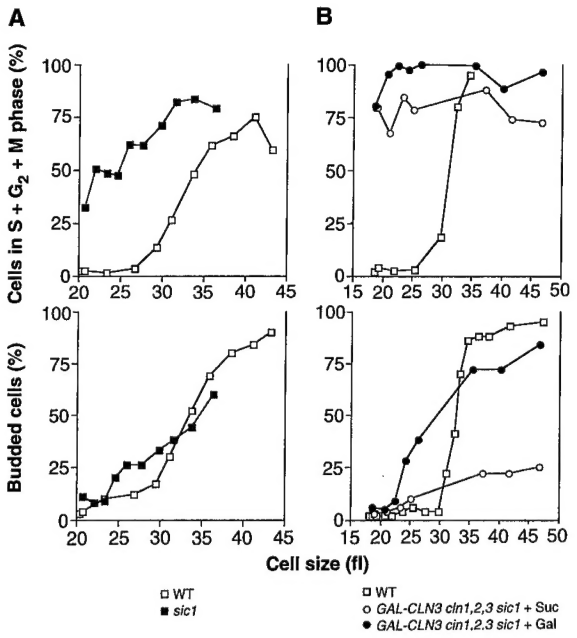

Fig. 4. A *sic1* deletion uncouples S phase from budding. (**A**) Small unbudded cells of strain W303a (*19*) (□) or its isogenic *sic1::URA3* derivative BS193 (■) were obtained by elutriation (*21*). Cells were reinoculated in fresh, warm medium, and samples were taken every 15 min and analyzed for budding, cell volume, and DNA content (FACS) (*22*). (**B**) Strain BS147 (*pGAL-CLN3 Δcln3 Δsic1*) (*19*) was grown in sucrose plus galactose. Cells were washed and resuspended in medium containing sucrose but no galactose to turn off *GAL-CLN3*. After 1 hour, small unbudded cells were collected by elutriation (*21*). Half the sample was reinoculated into YNB medium with 2% sucrose (*GAL-CLN3* off) (○), and the other half was reinoculated into YNB medium with 1% sucrose and 1% galactose (*GAL-CLN3* on) (●). Samples were taken every 30 min and analyzed as in (A). W303a cells (*19*) grown in YNB + 2% sucrose were elutriated and monitored after reinoculation (□).

replicated DNA much earlier than the wild-type cells, but budded at about the same time (Fig. 4A). [In other, similar experiments, the *sic1* mutation did advance budding slightly, although never as much as the advance in S phase (*2*, *11*). The early activation of Clb5 that occurs in *sic1* cells may advance budding.]

In a second experiment, *cln1 cln2 GAL-CLN3 sic1* cells were grown with *GAL-CLN3* on, and then *GAL-CLN3* was turned off for 1 hour. Small unbudded cells were obtained by elutriation. Fifty to 80% of these cells had a DNA content greater than 1N, despite their lack of Cln. (The large fraction of 2N cells probably resulted from overexpression of *CLB5* induced by *GAL-CLN3*.) When the cells were released into fresh medium, efficient budding was still dependent on reexpression of Cln3, whereas S phase was not (Fig. 4B). Thus, in *sic1* mutants, replication and budding are uncoupled; they occur at different times, and budding is much more dependent on Cln than is replication.

Although phosphorylation and loss of Sic1 are dependent on both Cln and Cdc34 function, we have not shown that Sic1 is a direct substrate of the Cln-Cdc28 kinase in vivo, nor that Sic1 proteolysis is ubiquitin-mediated. However, these are both strong possibilities. Phosphorylation converts at least one other protein into a substrate for Cdc34-mediated proteolysis (*12*). Whatever the precise mechanism by which Clns and Cdc34 cause the loss of Sic1, our genetic experiments show that this loss is largely responsible for the normal dependence of DNA replication on Start.

An analogous system may be used by mammalian cells. Cyclin D–Cdk4 complexes promote S phase by inhibiting function of the retinoblastoma protein. In cells lacking retinoblastoma, the cyclin D–Cdk4 activity is no longer required (*13*).

The identification of Sic1 as a target of Clns suggests that Start consists of several component events. The Start event controlling S phase is probably phosphorylation of Sic1; phosphorylation of other substrates may control budding and duplication of the spindle pole body, and together these phosphorylations constitute Start.

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6. Two-dimensional gel electrophoresis of Sic1 phosphorylated in vitro by Cdc28 showed 13 labeled charge isoforms, suggesting 13 phosphorylation sites. There are nine Ser-Pro or Thr-Pro sites in Sic1. Even the most highly phosphorylated Sic1 showed only a modest change in mobility in the SDS-PAGE dimension, consistent with the data shown in Figs. 1 and 2.
7. Sic1 from cells arrested in various states was treated with phosphatase as in Fig. 1 to show that the mobility shift was due to a change in phosphorylation. Consistent with these results, the mobility of Sic1 is altered in *cdc28* mutants (*2*).
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14. Antibody to rabbit Sic1 (12.5 μl) [J. D. Donovan, J. H. Toyn, A. L. Johnson, L. H. Johnston, *Genes Dev.* **8**, 1640 (1994)] was added to a 3-mg cell extract. After incubation for 1 hour at 0°C, protein A beads (30 μl) were added, and the mixture was rocked at 4°C for 1 hour. Beads were washed four times with alkaline phosphatase buffer (APB) [50 mM tris-HCl (pH 8), 10 mM dithiothreitol, 0.6 mM dimethylaminopurine, 1 mM phenylmethylsulfonyl fluoride, leupeptin (5 μg/ml), tosyl-L-phenylalanine-chloromethyl ketone (10 μg/ml), pepstatin (5 μg/ml), and soybean trypsin inhibitor (10 μg/ml)].
15. Extract (50 μg) was loaded per lane on a 16 cm by 18 cm by 0.75 mm gel and run for 20 hours at 100 V.
16. Proteins were transferred to nitrocellulose for 30 min at 10 V. Blots were blocked by using non-fat milk (5%) in tris-buffered saline [TBS; 140 mM NaCl, 2.5 mM KCl, 25 mM tris-HCl (pH 7.4)] for 1 hour. Blots were incubated overnight with a 1:100 dilution of rabbit antibody to Sic1 (*14*). Blots were washed four times in TBS, then incubated with a 1:2000 dilution of alkaline phosphatase–conjugated goat antibody to rabbit immunoglobulin G (Pierce) for 1.5 hours, washed, and finally incubated at room temperature with 10 ml of NBT-BCIP (nitroblue tetrazolium chloride/5-bromo-4-chloro-3-indolphosphate *p*-toluidine) (Gibco-BRL) for 5 to 10 min. β-Tubulin was used as a loading control.
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18. Beads (30 μl) carrying immunoprecipitated Sic1 (*14*) were divided into three portions (10 μl), and these were treated with APB (10 μl) (*14*), APB (8 μl) plus calf intestinal phosphatase (CIP) (2 μl, 2 U) (Boehringer), or APB (7 μl), CIP (2 μl), and B-glycerolphosphate (1 μl of 1 M).
19. Strains were W303a (*MAT***a** *ade2 his3 leu2 trp1 ura3 can1-100 ssd1-d [psi$^+$]*) [B. J. Thomas and R. Rothstein, *Cell* **56**, 619 (1989)], #31 (*MATα cdc34-2 cln1::HIS3 cln2::TRP1 ura3::GAL-CLN3 leu2 ura3*), BS100 (*MATα cln1::LEU2-GAL-CLN1-HA3 cln2::TRP1 cln3::HIS3 leu2 his3 ura3 ade2 trp1*), BS147 (*MAT***a** *cln1 cln2 cln3 sic1::TRP1* [*pGAL-CLN3 CEN URA3*] *ura3 leu2 trp1 his2 ade1*), BS152 (*cln1 cln2 cln3 sic1::TRP1*) (derived from BS147 by plasmid loss), and BS178 [(*MAT***a** *cln1::LEU2-GAL-CLN1-HA3 cln2 cln3 sic1::TRP1 ura3 trp1 his*(2 or 3)]. The *sic1::TRP1* allele was from M. Tyers; the parent of BS147 was from F. Cross.
20. Cells were grown to 1×10^7 cells per milliliter. The galactose medium was YEP (1% yeast extract, 1% peptone) with 1% raffinose and 1% galactose; the glucose medium was YEP with 1% raffinose and 2% glucose. Before shifting from one medium to another, cells were first washed twice with the new medium that had been prewarmed to the target temperature. Sic1 was detected as described (*15*, *16*). Representative samples were treated with phosphatase as shown (Fig. 1) to demonstrate that the mobility shift was due to phosphorylation.
21. Cells were grown in SD medium [F. Sherman, *Methods Enzymol.* **194**, 3 (1991)] with required amino acids to 2×10^7 cells per milliliter with 2% filter-sterilized sucrose (W303 and BS193) or 1% filter-sterilized sucrose plus 1% galactose (BS147). Cells were centrifuged, sonicated, and elutriated in medium at 30°C.
22. A computer curve-fitting algorithm estimated the number of cells with DNA content of 1N, 2N, or between 1N and 2N.

23. We thank L. Johnston for antibody to Sic1; M. Mendenhall, M. Tyers (who independently found suppression of the *cln1 cln2 cln3* mutant by *sic1*), and F. Cross for strains, reagents, helpful discussions, and communication of unpublished results; and M. Cleary and M. Luke for reading the manuscript. Supported by NIH grant GM 39978 and U.S. Army Breast Cancer grant DAMD17-94-J-4050 to A.B.F.

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